

A Comparative Study of the Intact Mass, Subunit Mass, and Released Glycans of Two Rituximab Biosimilars Using High-Resolution LC/MS

Author

Brian Liau
Agilent Technologies, Inc.

Introduction

Recent years have witnessed a rapid increase in the development and manufacture of biosimilar monoclonal antibodies (mAbs), driven by the expiry of patent protection for several highly profitable innovator biologics. Although there is no world-wide regulatory standard for biosimilars, there is consensus that biosimilars should: (i) possess a high degree of biophysical and biochemical similarity to the innovator biologic, and (ii) show comparability in purity, potency, and safety.¹ Regulatory agencies such as the US FDA have said that high-quality analytical data may be used to justify *in vivo* studies that are narrower in scope,² underscoring the importance of comprehensive, high-resolution analytical characterization.

Unlike small molecule drugs, mAbs are exceedingly complex, making complete analytical characterization difficult. Consequently, biopharmaceutical firms will specify a set of Critical Quality Attributes (CQAs) to be monitored, which have been selected based on evidence that they materially affect the overall quality, safety, or efficacy of the biologic. As several therapeutics may be produced in the same plant, one CQA is the mAb's identity, which is most efficiently determined by intact protein mass spectrometry. In addition, glycosylation is frequently considered a CQA because nonhuman glycan motifs can trigger acute or chronic allergic reactions.^{3,4} In addition, as mAbs are produced by cultured cells, glycans are highly sensitive to changes in culture conditions, making them useful indicators of unintended alterations to the manufacturing process.

This application note analyzes the intact mass, subunit mass, and glycosylation of two biosimilars of Rituximab, and compare them to the innovator molecule. We perform the analyses using four different workflows: (i) intact mAb, (ii) reduced subunits, (iii) IdeS-digested subunits, and (iv) released glycans. These workflows range in sample preparation time, from no sample preparation (intact mAb) to approximately three hours (released glycans). The advantage of workflows with more sample preparation is often a higher sensitivity to low abundance glycan components, increasing the analytical power of the technique.

We show that Agilent's high-resolution LC/MS, comprising the Agilent 1290 Infinity II LC system and the Agilent 6545XT Q-TOF, is well suited to performing these analyses. In addition, the AssayMAP Bravo is used to automate sample preparation for the released glycan workflow, reducing human error and sample preparation time. Labeled glycans are eluted at high concentration in small sample volumes, obviating the need for lengthy drying steps.

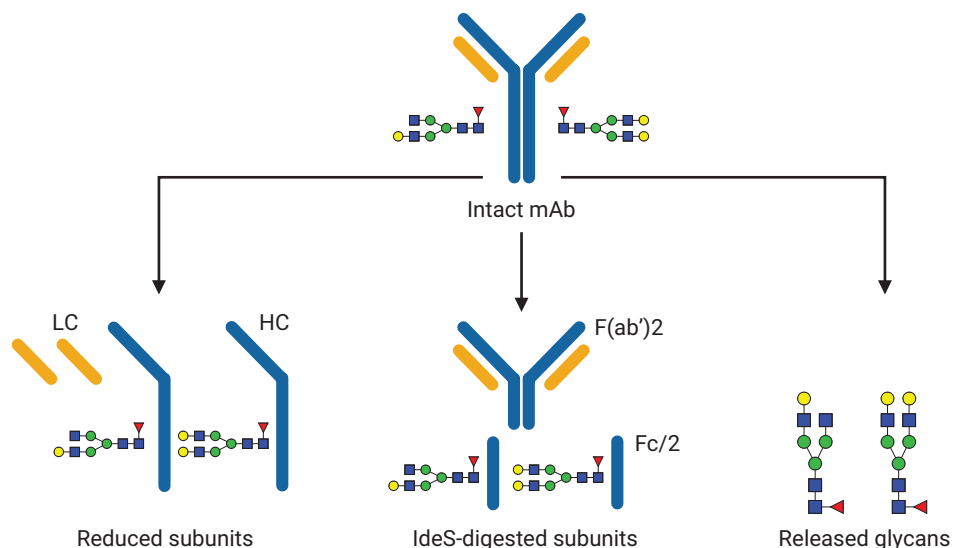


Figure 1. Workflows performed in this application note.

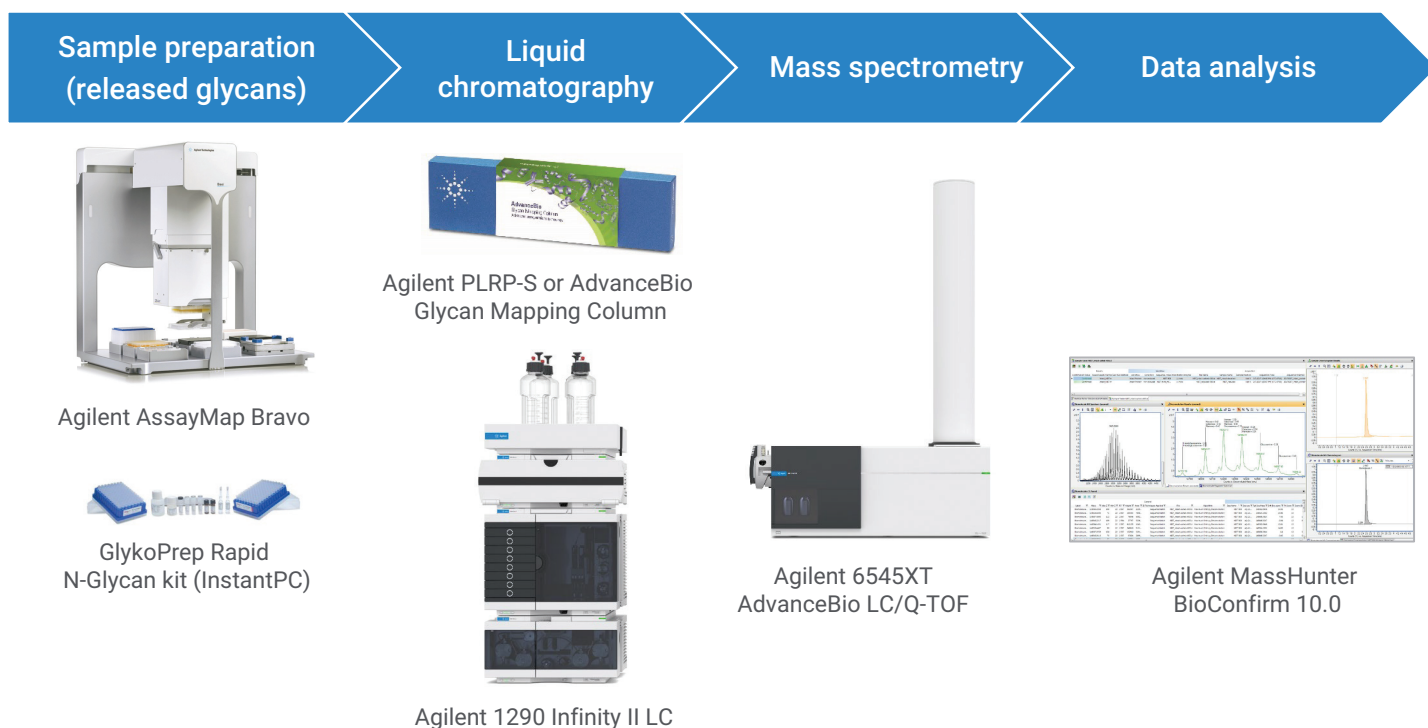


Figure 2. Equipment and consumables used in this application note.

Experimental

Materials and methods

Biosimilar and innovator versions of Rituximab were purchased from a local distributor in Singapore. Dithiothreitol (DTT), guanidine HCl, formic acid, ammonium formate, acetonitrile, ammonium bicarbonate, and Tris-HCl were purchased from Sigma. IdeS protease was purchased from Genovis. All mobile phase components were LC/MS grade.

Instrumentation

- Agilent AssayMAP Bravo system (G5542A) – included for released glycan experiments only
- Agilent 1290 Infinity II LC system including:
 - Agilent 1290 Infinity II High Speed Pump (G7120A)
 - Agilent 1290 Infinity II Multisampler (G7167B)
 - Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
 - Agilent 1260 Infinity Fluorescence Detector (G1321B) – included for released glycan experiments only
- Agilent 6545XT AdvanceBio LC/Q-TOF

Sample preparation

For intact mAb experiments, 10 mg/mL samples were analyzed without further sample preparation.

For reduced mAb heavy chain subunit experiments, samples were diluted to 1 mg/mL in freshly prepared 50 mM ammonium bicarbonate (pH 8.0) containing 4 M guanidine HCl. Samples were heated to 56 °C for 60 minutes, and allowed to cool to room temperature before analysis.

For IdeS-digested Fc/2 subunit experiments, IdeS protease was reconstituted in 50 mM Tris-HCl (pH 7.5) to a concentration of 2 Units/μL. mAb samples were diluted to 2 mg/mL in Tris-HCl buffer. Five microliters of IdeS protease were then added to 5 μL of each mAb sample, incubated at 30 °C for 30 minutes, then allowed to cool to room temperature before analysis.

For released glycan experiments, the AssayMap Bravo liquid handling system (G5542A) was used in conjunction with an Agilent GlykoPrep Rapid N-Glycan kit (GPPNG-PC) according to the instructions provided with the kit. Each mAb was processed in duplicate samples of 50 μg. After the final cleanup step, labeled glycans from each sample were eluted in volumes of 50 μL. To improve peak shape, 70 μL of acetonitrile

was added to 30 μL of labeled glycans from each sample before analysis. Injections were made in triplicate from each sample.

LC/MS analysis

Chromatographic separation of intact mAb, reduced mAb subunits, and IdeS-digested subunits were performed on an Agilent PLRP-S column (2.1 × 50 mm, 1000 Å, 5 μm). The flow was directed to waste during the first one minute of every chromatographic run. For intact mAb, the sample peak eluted at 2.4 minutes; for reduced mAb subunits, the light chain eluted at 1.8 minutes, and the heavy chain at 2.1 minutes; for IdeS-digested subunits, Fc/2 eluted at 1.3 minutes, and F(ab')₂ eluted at 2.1 minutes.

InstantPC-labeled glycans were chromatographically separated on an Agilent AdvanceBio Glycan Mapping column (2.1 × 150 mm, 1.8 μm). The fluorescence detector was set to Ex/Em = 285 nm/345 nm, with PMT gain = 10. Tables 1 and 2 list the parameters used. To reduce in-source fragmentation of glycans, SWARM autotune with the “fragile ions” option was performed prior to analysis. Relative quantitation of glycans was performed by comparing peak areas of the fluorescence chromatogram.

Table 1. Chromatographic parameters.

Agilent 1290 Infinity II LC System				
Sample Type	Intact mAb	Reduced mAb subunits (HC and LC)	IdeS mAb subunits (Fc/2 and F(ab') ₂)	Released glycans
Column	Agilent PLRP-S, 2.1 × 50 mm, 1000 Å, 5 μm (p/n PL1912-1502)	Agilent PLRP-S, 2.1 × 50 mm, 1000 Å, 5 μm (p/n PL1912-1502)	Agilent PLRP-S, 2.1 × 50 mm, 1000 Å, 5 μm (p/n PL1912-1502)	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 μm (p/n 859700-913)
Solvent A	0.1% Formic acid in DI water	0.1% Formic acid in DI water	0.1% Formic acid in DI water	50 mM Ammonium formate, pH 4.5
Solvent B	0.1% Formic acid in acetonitrile	0.1% Formic acid in acetonitrile	0.1% Formic acid in acetonitrile	Acetonitrile
Gradient	0 to 1 minutes, 0 to 20% B 1 to 3 minutes, 20 to 50% B 3 to 4 minutes, 50 to 70% B	0 to 5 minutes, 25 to 45% B 5 to 6 minutes, 45 to 60% B 6 to 7 minutes, 60% B	0 to 1 minutes, 25% B 1 to 6.5 minutes, 25 to 60% B 6.5 to 7.5 minutes, 60% B 7.5 to 7.6 minutes, 60 to 25% B 7.6 to 8.5 minutes, 25% B	0 to 2 minutes, 25 to 30% B 2 to 15.5 minutes, 30 to 37% B 15.5 to 17 minutes, 37 to 80% B 17 to 20 minutes, 80% B 20 to 21 minutes, 80 to 25% B 21 to 30 minutes, 25% B
Column Temperature	60 °C	60 °C	60 °C	40 °C
Flow Rate	0.5 mL/min	0.8 mL/min	0.5 mL/min	15.5 to 20 minutes: 0.25 mL/min Otherwise: 0.4 mL/min
Injection Volume	0.5 μL	1 μL	1 μL	2 μL

Table 2. Mass spectrometry parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System				
Sample Type	Intact mAb	Reduced mAb subunits (HC and LC)	IdeS mAb subunits (Fc/2 and F(ab') ₂)	Released glycans
Source	Agilent Dual Jet Stream			
Gas Temperature	350 °C	350 °C	350 °C	150 °C
Gas Flow	12 L/min	12 L/min	12 L/min	9 L/min
Nebulizer	60 psig	35 psig	35 psig	35 psig
Sheath Gas Temperature	400 °C	350 °C	350 °C	300 °C
Sheath Gas Flow	11 L/min	11 L/min	11 L/min	10 L/min
Vcap	5.5 kV	4 kV	4 kV	3 kV
Nozzle Voltage	2 kV	2 kV	2 kV	500 V
Fragmentor	380 V	180 V	180 V	120 V
Skimmer	140 V	65 V	65 V	65 V
Mass Range	100 to 10,000 <i>m/z</i>	100 to 3,200 <i>m/z</i>	800 to 5,000 <i>m/z</i>	300 to 1,700 <i>m/z</i>
Acquisition Rate	1 spectrum/sec	1 spectrum/sec	1 spectrum/sec	2 spectra/sec
Reference Mass	922.0098			
Acquisition Mode	Positive, extended (10,000 <i>m/z</i>) mass range	Positive, standard (3,200 <i>m/z</i>) mass range, HiRes (4 Ghz)	Positive, extended (10,000 <i>m/z</i>) mass range	Positive, low mass range, HiRes (4 Ghz)

Data processing

Agilent MassHunter BioConfirm 10.0 software was used for all data analyses. For released glycans, fluorescence chromatograms were extracted using MassHunter Qualitative Analysis version 10.0, and glycan compositions were assigned using the Agilent Personal Compound Database and Library (PCDL) glycan database.⁵

For intact mAb, reduced mAb subunit, and IdeS-digested subunit analyses, Maximum Entropy deconvolution with a mass step of 1 Da was used.

For released glycans, match tolerances were set to 10 ppm. Allowed ion species were H⁺, Na⁺, K⁺, NH₄⁺ and H⁻, with a charge range state of 1 to 3. The InstantPC tag option was selected in the software.

Results and discussion

High resolution mass spectrometry of intact mAbs can be conducted with minimal sample preparation and used to rapidly assess the identity of the mAb and its major glycoforms. Most therapeutic mAbs contain a

single glycosylation site on each heavy chain, with the resulting glycoforms representing different combinations of two glycans. In addition, lysine residues on the C-termini of the heavy chains may be clipped to varying degrees depending on the amount of Carboxypeptidase D activity during cell culture.⁶

Table 3. Deconvolution settings.

Agilent MassHunter BioConfirm B10.0 Settings			
Sample Type	Intact mAb	Reduced mAb subunits (HC and LC)	IdeS mAb subunits (Fc/2 and F(ab') ₂)
Deconvolution	Maximum entropy		
Deconvolution <i>m/z</i> Range	2,000 to 6,500	800 to 2,600	1,000 to 2,600
Deconvolution Mass Range	140 to 160 kDa	20 to 60 kDa	21 to 28 kDa
Deconvolution Subtract Baseline	7.0	7.0	6.0
Match Tolerance	20 ppm	20 ppm	20 ppm

Figures 3A and 3B show deconvoluted mass spectra of each biosimilar mAb mirrored against the innovator mAb for comparison. The identities of the biosimilars and innovator mAb could be established with high confidence through comparison of their accurate masses. Using the G1F+G0F-2 Lys glycoform as a basis for comparison, biosimilar 1 was identified with 7.18 ppm error (147,240.79 Da), biosimilar 2 with 9.98 ppm error (147,241.22 Da), and the innovator with 4.78 ppm error (147,240.44 Da). Table 4 shows that except for 2*G0F-2 Lys, all other glycoforms in biosimilar 2 were matched at <10 ppm error.

Figure 3A shows that biosimilar 1 is more similar to the innovator mAb than biosimilar 2. In Figure 3B, biosimilar 2's spectrum is made more complex by the presence of varying degrees of C-terminal lysine truncation, each reflected by an average mass shift of -128.175 Da, causing each glycoform to be represented as three peaks instead of just one. The varied degrees of lysine truncation in biosimilar 2 has been confirmed in peptide mapping experiments performed in a different study.⁷

BioConfirm 10.0 software allows the user to perform relative quantitation on selected glycoforms. Figure 3C summarizes the relative quantity of each glycoform averaged over N = 6 observations. Biosimilar 2 has 8.3 ± 1.5% more G0F+G0F, 6.2 ± 1.9% more G0F+G1F, and 8.6 ± 4.3% less G2F+G2F glycoforms than the innovator, indicating that its glycan content deviates substantially from the innovator. Conversely, biosimilar 1's glycoforms are a closer match, with the main difference being 5.3 ± 1.5% more G0F+G1F.

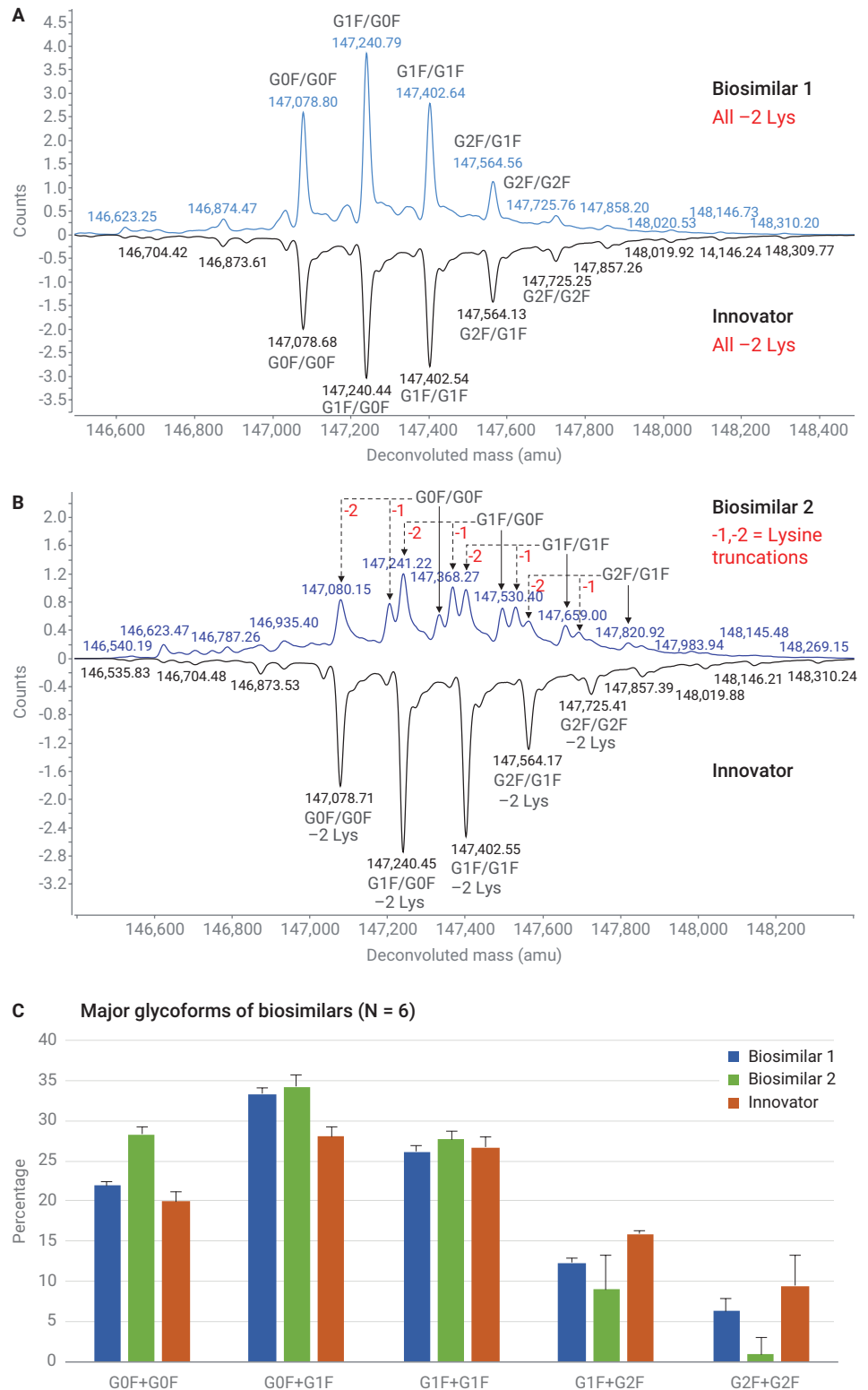
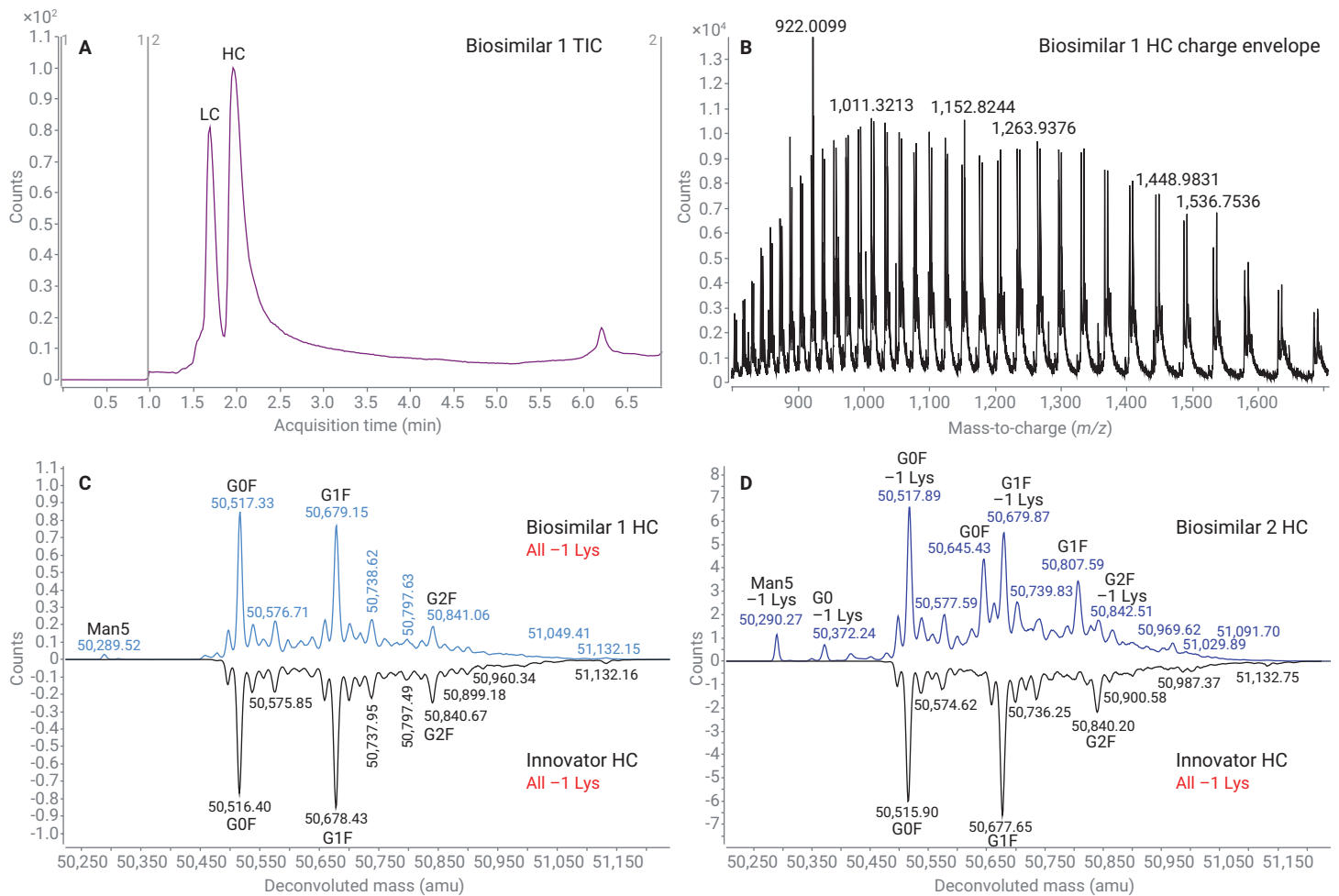


Figure 3. Deconvoluted intact mAb spectra showing major biosimilar glycoforms. (A) Biosimilar 1 mirrored against the innovator. All glycoforms include the loss of two C-terminal lysine residues (-2 Lys). (B) Biosimilar 2 mirrored against the innovator. Biosimilar 2 has varying degrees of lysine loss. (C) Summary statistics of glycoforms. Error bars represent standard deviations (N = 6).

The size and complexity of the sample may be lowered through subunit analysis to facilitate detection of low abundance glycans. Subunits may be generated by reducing disulfide bonds using agents such as DTT and *tris*(2-carboxyethyl) phosphine (TCEP), or by enzymatic cleavage using IdeS protease. Figure 4 shows deconvoluted mass spectra of biosimilar heavy chains (~50 kDa) produced by complete reduction of disulfide bonds with DTT. Figure 5 shows Fc/2 subunits (~25 kDa) resulting from digestion of samples with IdeS.

Table 4. Glycoforms matched by BioConfirm in biosimilar 2.

Biosimilar 2 Glycoforms				
Mass	Area (MS)	%Quant (Area)	Diff (Bio, ppm)	Predicted Modifications
147,080.15	25,527,902	14.01	17.34	2*G0F (NGA2F)(1,445.3580) + 2*Lys-loss(-128.1750)
147,205.55	770,401	6.99	-1.53	2*G0F (NGA2F)(1,445.3580) + 1*Lys-loss(-128.1750)
147,241.22	32,853,404	15.98	9.98	1*G1F(1,607.5013) + 1*G0F (NGA2F)(1,445.3580) + 2*Lys-loss(-128.1750)
147,333.70	7,733,970	5.52	-1.71	2*G0F (NGA2F)(1,445.3580)
147,368.27	25,443,047	9.2	2.35	1*G1F(1,607.5013) + 1*G0F (NGA2F)(1,445.3580) + 1*Lys-loss(-128.1750)
147,402.38	28,448,038	14.37	3.31	2*G1F(1,607.5013) + 2*Lys-loss(-128.1750)
147,496.45	96,402	6.55	2.38	1*G1F(1,607.5013) + 1*G0F (NGA2F)(1,445.3580)
147,530.40	28,606,318	6.83	2.29	2*G1F(1,607.5013) + 1*Lys-loss(-128.1750)
147,563.14	23,128,142	9.49	-6.05	1*G2F (NA2F)(1,769.6445) + 1*G1F(1,607.5013) + 2*Lys-loss(-128.1750)
147,659.00	19,354,473	4.69	5.18	2*G1F(1,607.5013)
147,692.91	47,347	3.79	4.73	1*G2F (NA2F)(1,769.6445) + 1*G1F(1,607.5013) + 1*Lys-loss(-128.1750)
147,820.92	14,880,698	2.6	3.63	1*G2F (NA2F)(1,769.6445) + 1*G1F(1,607.5013)



At the level of reduced heavy chain (HC) subunits shown in Figure 4, lower abundance glycans such as Man5 and G0 are observable. This is significant because Man5 has been shown to increase the clearance of therapeutic mAbs,⁸ and is often considered a critical quality attribute. In comparison to the innovator, Figure 4C shows that Man5 is increased in biosimilar 1, and Figure 4D shows that Man5 and G0 are both increased in biosimilar 2.

Since IdeS-digested Fc/2 subunits are approximately half the size of reduced HC subunits, their analysis provides even greater sensitivity for low abundance glycoforms. Figure 5 shows that Man5 is detectable in innovator Fc/2 subunits, and even low abundance biosimilar 2 glycoforms such as Man5 and G0 on subunits without lysine truncation can be distinguished clearly.

High-sensitivity glycan analysis can be achieved by releasing glycans using PNGase-F enzyme, followed by labeling with a fluorescent dye. This analysis requires intensive sample preparation, and would ordinarily take 8 to 16 hours with a great deal of manual intervention. We have developed an automated high-throughput workflow using the AssayMAP Bravo liquid handling system and Agilent GlykoPrep Rapid N-Glycan kit that reduces the sample preparation time to only three hours.⁹

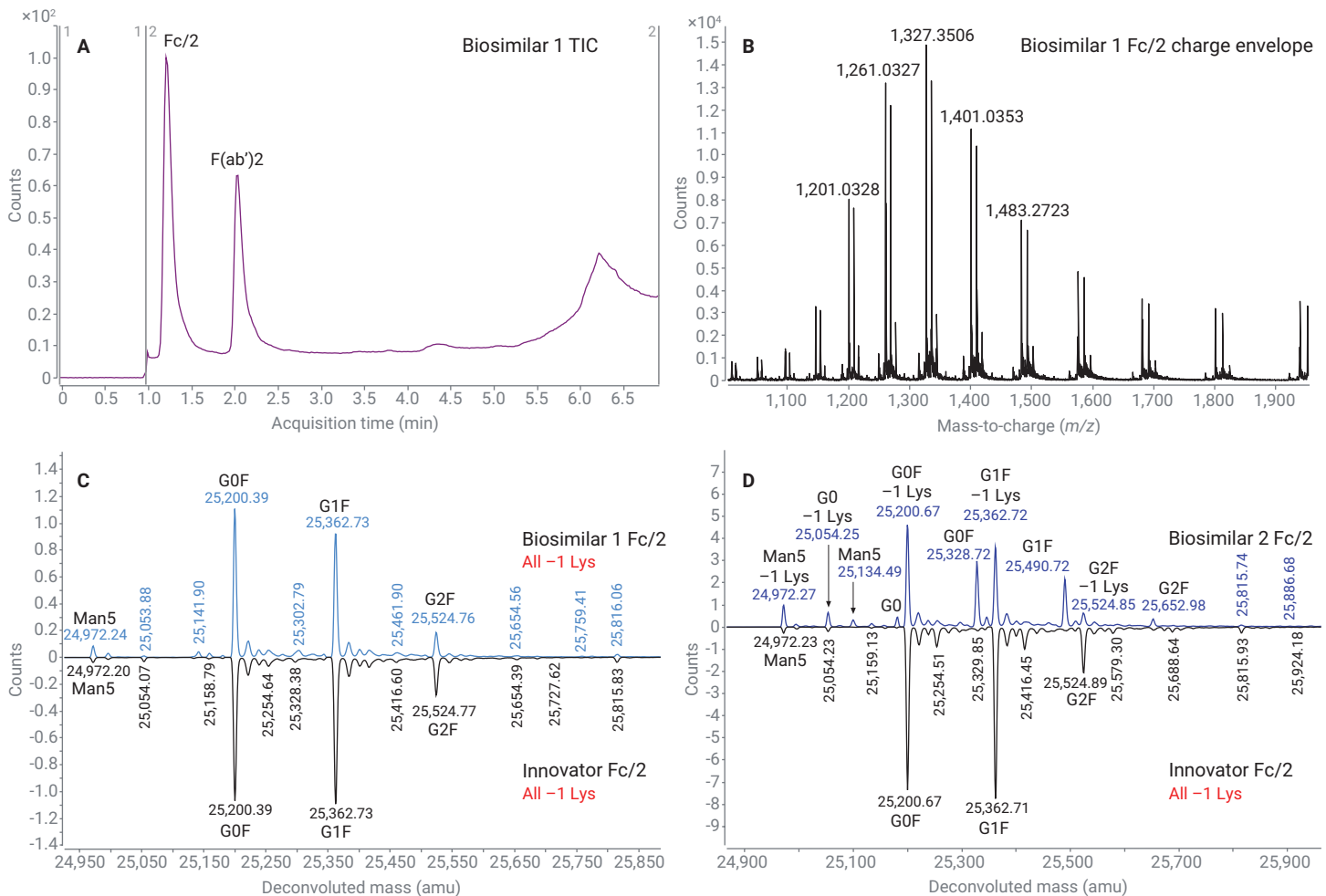


Figure 5. Deconvoluted mass spectra of IdeS-digested Fc/2 subunits. (A) TIC chromatogram showing separation of Fc/2 and F(ab')₂ in biosimilar 1. (B) Charge envelope of biosimilar 1 Fc/2 peak. (C) Biosimilar 1 Fc/2 mirrored against the innovator. All glycoforms include the loss of one C-terminal lysine (-1 Lys). (D) Biosimilar 2 Fc/2 mirrored against the innovator. Biosimilar 2 has varying degrees of lysine loss.

This is possible because the unique design of the liquid handling capillaries and sample reaction cartridges elute released glycans in a very small volume, eliminating time-consuming vacuum drying steps.

The fluorescent dye used in this experiment is InstantPC, a proprietary fluorophore that greatly enhances the ionization efficiency of glycans while reducing the time required for conjugation due to its unique chemistry.⁹

Figure 6 shows representative fluorescence chromatograms of biosimilar and innovator glycans separated on an AdvanceBio Glycan Mapping column, and Table 3 lists all observed glycans. Glycan compositions were assigned by BioConfirm 10.0 using accurate mass spectra, then annotated to fluorescence peaks by retention time correlation. Figure 6D shows that when assigning glycan identities, BioConfirm 10.0 matches signals and isotopic models from multiply charged

H^+ , NH_4^+ , Na^+ , and K^+ adduct ions, increasing confidence in the annotation. Coelutions were confirmed by manual inspection of extracted mass spectra. This analysis confirms that G0 and Man5 glycans are substantially increased in biosimilar 2 (Figure 6B), whereas G2F glycan is decreased in comparison to the innovator. In addition, biosimilar 2 contains appreciable quantities of afucosylated glycans H4N3 and H4N4, which were not detected in either biosimilar 1 or the innovator (Table 5).

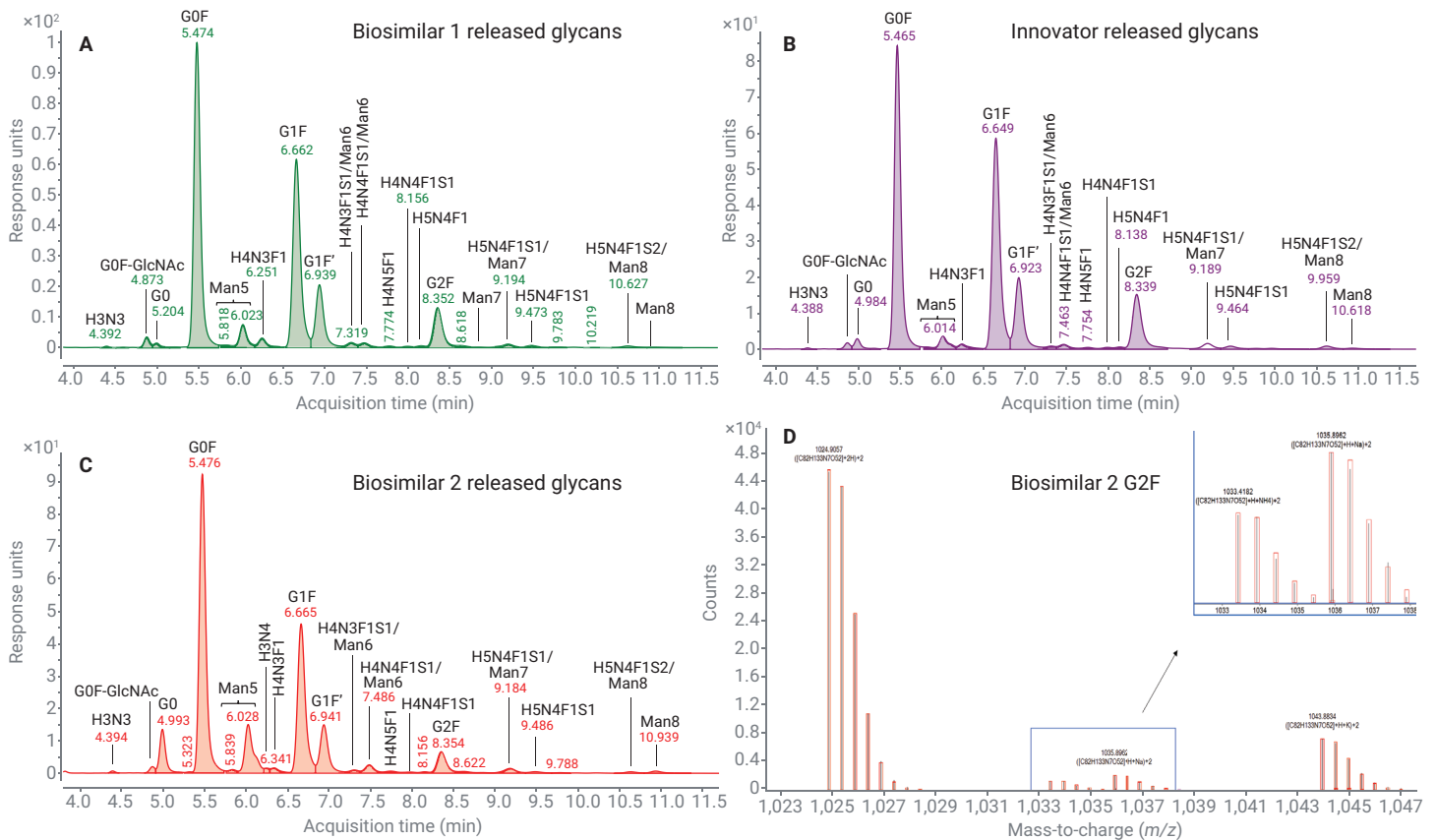


Figure 6. (A to C) Fluorescence chromatograms of InstantPC labelled glycans released from biosimilar and innovator samples. (D) Centroid mass spectrum of G2F glycan (RT = 8.35 minutes) in biosimilar 2. Red rectangles represent the expected isotopic model for each adduct. Note that BioConfirm 10.0 has successfully matched multiple adducts, including $[m+2H]^{2+}$, $[m+H+NH_4]^{2+}$, $[m+H+Na]^{2+}$, and $[m+H+K]^{2+}$.

Figure 7 illustrates the relative quantities of selected glycans estimated at the levels of reduced heavy chain subunits, IdeS-digested Fc/2 subunits, and released glycans. In general, higher analytical sensitivity is gained at the cost of longer sample preparation time and is accompanied by the loss of information as the protein's structure is progressively degraded. For example, Man5 and G0 glycans were not detected with intact mAb analysis (Figure 3) but were detected and quantified in subunit and released glycan analyses with progressively greater accuracy. This is due to the reduction in sample complexity through elimination of matrix effects from the protein or sample buffer, resulting in greater sensitivity to lower abundance glycoforms or released glycans.

Taken together, the data in this application note show that subunit analysis provides a level of sensitivity to lower abundance glycoforms that is intermediate between intact mAb and released glycan analyses, and may provide sufficient sensitivity to discriminate biosimilars with glycan compositions very different from the innovator. However, released glycan analysis is required for comprehensive glycan profiling, which enables subtle differences in low abundance glycans to be quantified accurately.

Table 5. Compositions and relative quantities of released glycans (N = 6).

Glycan	RT (min)	Biosimilar 1		Biosimilar 2		Innovator	
		Average	Std. Dev.	Average	Std. Dev.	Average	Std. Dev.
H3N3	4.392	0.15	0.04	0.25	0.07	0.10	0.06
G0F-GlcNAc	4.874	1.25	0.04	0.72	0.05	0.66	0.03
G0	4.995	0.62	0.05	5.58	0.19	1.23	0.04
H4N3	5.324	-	-	0.14	0.02	-	-
G0F	5.479	40.47	0.69	41.03	0.82	37.49	0.61
Man5	5.845	0.44	0.01	0.55	0.09	0.29	0.02
Man5	6.032	3.52	0.03	8.64	0.06	2.21	0.04
H4N3F1	6.255	1.68	0.01	0.52	0.04	1.01	0.06
H4N4	6.347	-	-	0.87	0.11	-	-
G1F	6.671	28.72	0.22	23.40	0.41	30.23	0.19
G1F'	6.946	10.38	0.14	8.18	0.11	11.28	0.05
H4N3F1S1 Man6	7.315	0.92	0.02	0.59	0.08	0.51	0.02
H4N4F1S1/Man6	7.495	0.94	0.02	1.58	0.10	0.94	0.03
H4N5F1/H5N4	7.754	0.30	0.01	0.45	0.09	0.35	0.03
H4N4F1S1	7.994	0.22	0.01	0.22	0.06	0.30	0.03
H5N4F1	8.167	0.28	0.01	0.19	0.09	-	-
G2F	8.363	7.29	0.20	4.10	0.09	9.84	0.15
Man7	8.854	0.14	0.01	0.11	0.05	0.05	0.07
H5N4F1S1/Man7	9.194	0.89	0.03	1.19	0.09	1.19	0.05
H5N4F1S1	9.495	0.50	0.02	0.37	0.06	0.67	0.03
H5N4F1S2/Man8	10.649	0.41	0.03	0.32	0.03	0.67	0.08
Man8	10.951	0.09	0.01	0.51	0.03	0.26	0.01

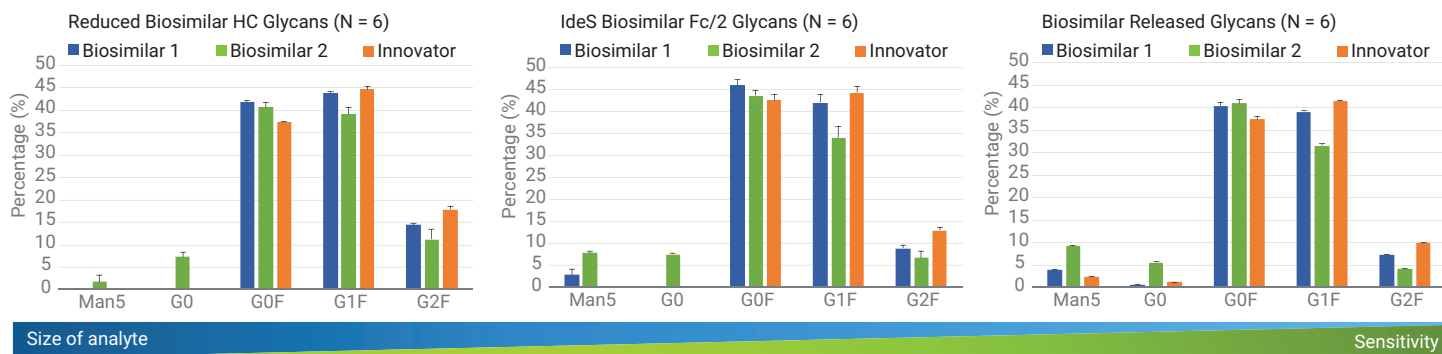


Figure 7. Relative quantities of selected glycans estimated by analysis of reduced HC subunits, IdeS-digested Fc/2 subunits, or released glycans. Error bars represent standard deviations (N = 6).

Conclusion

We have performed comprehensive analysis of two biosimilars and an innovator mAb using an Agilent 1290 Infinity II LC system, high-resolution Agilent 6545XT AdvanceBio LC/Q-TOF, and Agilent MassHunter BioConfirm 10.0 software. Intensive sample preparation steps were automated and expedited using Agilent AssayMap Bravo liquid handling technology for greater productivity and reproducibility.

We have implemented four analytical approaches that can be adapted to the needs of different stages of biopharmaceutical production:

- The intact mAb approach can rapidly assess the identity of and major glycoforms present in a sample, and is well suited for lot release and screening CHO cell clones in discovery/development. Almost no sample preparation is required with this approach, and unique higher-order information such as the relative quantities of glycoforms resulting from pairing differently glycosylated or lysine truncated heavy chains can be obtained.
- The reduced HC and IdeS-digested subunit approaches provide a level of sensitivity intermediate between intact mAb and released glycan approaches, and are well suited to routine at-line monitoring of bioreactor products in manufacturing.¹⁰ The higher level of sensitivity enables some low abundance CQAs such as Man5 to be detected and quantified.

- The released glycan approach provides the highest level of sensitivity, and is well suited to therapeutic development activities in discovery/development. Since this method relies on fluorescence chromatography, it is the most robust method for relative glycan quantitation of the four approaches. InstantPC is much brighter than traditional labels such as 2-AB, and covalently bonds with glycans in a 1:1 stoichiometry, enabling subtle differences in glycosylation to be detected and quantified.

We detected differences in glycosylation patterns between the two biosimilars and the innovator, namely the relative expression of G0F, G1F, and G2F, that were consistent across all four approaches used, illustrating the robustness and versatility of Agilent's LC/MS technologies for biosimilar development.

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